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Mouse muscle nicotinic acetylcholine receptor γ subunit: cDNA sequence and gene expression

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ABSTRACT

Clones coding for the mouse nicotinic acetylcholine receptor (AChR) γ subunit precursor have been selected from a cDNA library derived from a mouse myogenic cell line and sequenced. The deduced protein sequence consists of a signal peptide of 22 amino acid residues and a mature γ subunit of 497 amino acid residues. There is a high degree of sequence conservation between this mouse sequence and published human and calf AChR γ subunits and, after allowing for functional amino acid substitutions, also to the more distantly related chicken and *Torpedo* AChR γ subunits. The degree of sequence conservation is especially high in the four putative hydrophobic membrane spanning regions, supporting the assignment of these domains. RNA blot hybridization showed that the mRNA level of the γ subunit increases by 30 fold or more upon differentiation of the two mouse myogenic cell lines, BC3H-1 and C_2C_{12}, suggesting that the primary controls for changes in gene expression during differentiation are at the level of transcription. One cDNA clone was found to correspond to a partially processed nuclear transcript containing two as yet unspliced intervening sequences.

INTRODUCTION

The nicotinic acetylcholine receptor (AChR) on the postsynaptic membrane is an integral membrane protein complex composed of four subunits, α, β, γ, and δ. It functions as an agonist gated ion channel in the *Torpedo* electric organ and at the neuromuscular junction of striated muscle in other vertebrates. At present AChR is the best studied and most fully characterized ion channel. Related receptors are also present in the nervous system. The receptor is very abundant in the electric organ of the electric ray *Torpedo* and it has been extensively characterized at the biochemical, functional, and sequence levels (1-3). The amino acid sequences of the four *Torpedo* AChR subunits have been deduced from the nucleotide sequences of full-length cDNA clones (4-9). In vertebrate striated muscle and various muscle-like cell systems in culture, nicotinic AChR molecules are present in lower overall abundances. Vertebrate systems are of greater interest than *Torpedo* for most electrophysiological studies and for cell biological studies of assembly (10). The subunits of the vertebrate AChR are similar in general properties to those of *Torpedo*, but they are clearly somewhat divergent at the amino acid sequence level (11). The mouse myogenic cell line BC3H-1 (12) is one of the more abundant sources of mammalian AChRs and their mRNAs. Our

laboratory and others have described the isolation of cDNAs for several of the mouse AChR subunits from this source (13-15).

In particular, our laboratory has previously reported on the preparation of a cDNA library from membrane bound polysomal poly(A)$^+$ RNA of induced BC3H-1 cells (14). When this cDNA library was screened by low stringency hybridization with a *Torpedo* AChR γ chain probe, two groups of non-overlapping clones were isolated. A full sequence determination (14) and expression studies in *Xenopus* oocytes (16) led to the surprising conclusion that one of these groups of clones, selected by hybridization with a *Torpedo* γ probe, actually coded for a δ subunit. In the present paper, we report the sequence determination of the clones from the other group and show that this group does in fact code for the mouse AChR γ subunit. Also reported here are some features of γ subunit gene expression at the mRNA level.

MATERIALS AND METHODS

Chemicals and Reagents

Restriction endonucleases and other enzymes including T4 DNA ligase, Klenow fragment of E. coli DNA polymerase, SP6 and T7 RNA polymerases, exonuclease III and S1 nuclease were purchased from Bethesda Research Laboratories, Boehringer Mannheim Biochemicals, New England Biolabs, Promega Biotec, and Sigma.

Cloning of Mouse AChR γ cDNA and DNA Sequence Determination

A cDNA library was prepared in the vector λgt10 using membrane-associated polysomal polyadenylated RNA from differentiated BC3H-1 cells and screened with a *Torpedo* AChR γ cDNA probe as previously described (14). The screening was carried out at 42°C for 48 hours in the hybridization solution containing 33% (v/v) formamide, 0.9M NaCl, 50mM sodium phosphate, 5mM EDTA, pH 7.4, 0.1% Ficoll, 0.1% polyvinylpyrolidone, 0.1% bovine serum albumin, 0.1% sarcosine, 0.1 mg/ml denatured salmon sperm DNA, and 2μg/ml each of poly(rA), poly(rC), and poly(rG). Filters were washed in 30mM NaCl, 3mM sodium citrate, and 0.05% sarcosine at 50°C. cDNA inserts from plaques that gave positive signals were separated from the λgt10 arms by *Eco*RI restriction digestion and recloned either into the plasmid vector pUC19 (17) for restriction mapping, or into an M13 vector (18) for exonuclease III deletion (19) and subsequent sequence determination, or into the expression vector pIBI76 (International Biotechnology Inc.) for SP6 and/or T7 *in vitro* transcription.

For transformation of JM109 cells (17) with M13 RF DNA, Hanahan's protocol (20) was used with the following transformation buffer: 30mM NaAc, 30mM $CaCl_2$, 100mM KCl, 70mM $MnCl_2$, adjusted to pH5.6 with 0.1M acetic acid, and filter sterilized.

For sequence determination, full length cDNA clones or ExoIII-generated deletion clones were analyzed by the method of dideoxy nucleotide chain termination (21) with a few modifications (22).

Cell Culture

Cells were grown and fed every other day in DME (Dulbecco modified Eagle medium) supplemented with 20% fetal calf serum and Pen-Strep (100 units penicillin G/ml and 100 mcg streptomysin/ml) for BC3H-1 cells and 20% fetal calf serum and 0.5% chick embryo extract for C_2C_{12} cells. They were plated at a density of 2-2.5x10^4 cells/ml and propagated in a humidified 37°C incubator with 5% CO_2/95% air. Cells reached 40-60% confluence 2 days after plating and were harvested as undifferentiated cells. To induce differentiation, BC3H-1 cells were grown to confluence without feeding and harvested 6 days after plating. Similarly, C_2C_{12} cells were grown to confluence (4 days after plating), switched to DME medium supplemented with 2% horse serum, fed with this medium every day, and harvested 7 days after plating.

Generation of ^{32}P-labeled RNA Probes by *in vitro* Transcription

Double-stranded plasmid DNA bearing the desired insert was linearized with appropriate restriction enzymes, extracted with phenol-chloroform, precipitated with ethanol, and used as template in *in vitro* transcription. For SP6 or T7 transcription, 0.5µg of linear DNA was suspended in 20µl transcription solution (40mM Tris, pH7.9, 6mM $MgCl_2$, 2mM spermidine, 10mM DTT, 0.5mM each of ATP, GTP and UTP, 100µCi [^{32}P-α]CTP with a specific activity of 410Ci/mM, and 20 units of RNasin). 0.5µl of SP6 or T7 RNA polymerase was added, and the reaction carried out at 37°C for 60 minutes. 10µl of 1mg/ml yeast tRNA and 1µl of 1mg/ml RNase-free DNase was added, and the mixture incubated at 37°C for another 15 minutes. The mixture was then passed over a Sephadex G-50-80 column to remove the unincorporated nucleotide precursors. The radioactive RNA solution from the column was used in hybridization without further treatment.

RNA Purification, Fractionation, Blotting, and Hybridization

Cultured cells were lysed by 6M guanidine solution (6M guanidine-HCl, 0.2M NaAc, 0.1M β-mercaptoethanol, pH4.6). The lysate was homogenized manually in a glass homogenizer to reduce viscosity, and the RNA precipitated with 0.5 volumes of pure ethanol. The pellet was dissolved in 7.5M guanidine solution (7.5M guanidine-HCl, 25mM sodium citrate, 50mM β-mercaptoethanol, pH6.4), and the RNA precipitated with 0.025 volumes of 1M acetic acid and 0.5 volumes of pure ethanol. This pellet was dissolved in 0.1% SDS solution and extracted twice with equal volume of phenol-chloroform and twice with chloroform; the aqueous solution was transferred to a glass tube containing 0.1 volumes of 3M NaAc (pH4.8) and 2.5 volumes of pure ethanol; and the RNA was precipitated. The RNA pellet was dissolved in 0.05% SDS solution and precipitated again with 0.1 volumes of 3M NaAc and 2.5 volumes of pure ethanol. Finally the RNA was resuspended in 0.05% SDS solution and stored at -80°C.

For gel blots, RNA was fractionated on a glyoxal gel (23), blotted to Hybond-N

membrane (Amersham) in 20XSSC (3M NaCl, 0.3M Na-citrate) by capillary action for 18 hours, and crosslinked to the membrane by irradiation with a standard UV transilluminator for 5 minutes.

RNA blots were prehybridized for 4 hours at 60°C in hybridization solution (0.1M Na_2HPO_4/NaH_2PO_4, pH6.5, 5XSSC, 2.5X Denhardts, 50% formamide, 0.1% SDS, 1.25mM EDTA, 0.1 mg/ml denatured calf thymus DNA, 0.1 mg/ml yeast tRNA), ^{32}P-labeled RNA probe was added to 10^6cpm/ml, and the hybridization was carried out at 60°C for 16 hours. The blots were washed at 65°C three times in 2XSSC, 0.1%SDS and three times in 0.2XSSC, 0.1%SDS, wrapped in plastic sheets, and exposed to X-ray films (Kodak XAR-5).

RESULTS AND DISCUSSION

The Mouse cDNA Clones

As previously reported (14), a cDNA library was constructed in the vector λgt10 using membrane-bound polysomal polyadenylated RNA prepared from differentiated BC3H-1 cells, a relatively rich source of AChR. By screening the library at low stringency with a full length cDNA clone for the AChR γ subunit of the electric ray *Torpedo californica*, two groups of non-overlapping clones were isolated. Surprisingly, one group of clones, selected with the *Torpedo* γ probe, turned out to code for the mouse δ subunit, even though there was no detectable hybridization in screening the library with a *Torpedo* δ probe (14). In the work reported here, we have studied the other group of clones selected by hybridization with the *Torpedo* γ probe. Restriction endonuclease mapping analysis of these inserts revealed that they formed a group of

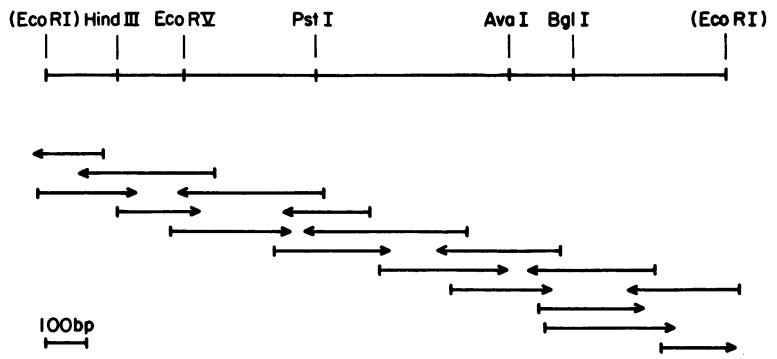

Figure 1. Restriction map of clone M169 and the exonuclease III-generated deletion clones. The restriction map of clone M169 is shown on top. The *Eco*RI sites flanking the clone were added during the cDNA library construction. The exonuclease III-generated deletion clones are aligned below and the regions sequenced are indicated by the arrowed bars.

overlapping clones (data not shown).

Nucleotide Sequence Determination and Assignment of the Protein Sequence

One of the longest cDNA clones, M169, was selected for sequence analysis. The insert was recloned into the *Eco*RI site of the M13mp18 vector and transformed into the *E. coli* host JM109. Clones containing the insert in both orientations were selected and treated with exonuclease III to generate controlled deletions (19). The restriction map of clone M169 is shown in Figure 1 and the ExoIII-generated deletion clones for sequencing are aligned below it. Sequence analysis was carried out by the dideoxy

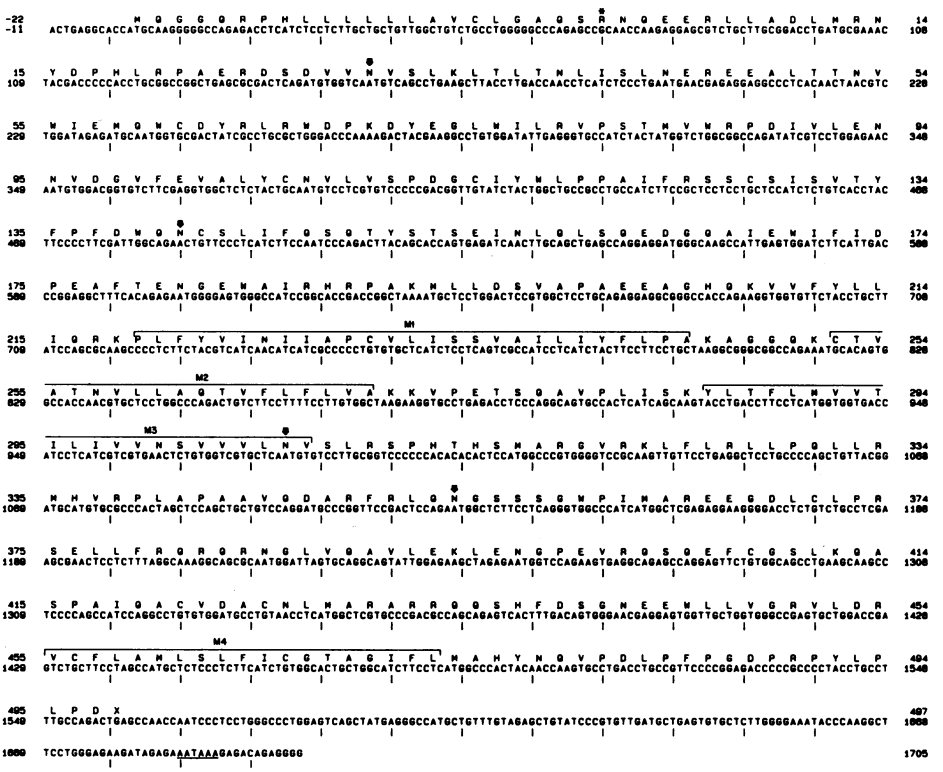

Figure 2. Nucleotide and deduced amino acid sequence of the mouse AChR γ subunit precursor. Nucleotide no. 1 indicates the first nucleotide of the initiation codon in the protein coding region, and the nucleotides 5' to the initiation codon are indicated by negative numbers. The sequence shown is followed on its 3' side by a stretch of adenosine residues (not shown). The putative polyadenylation signal sequence AATAAA is underlined. The deduced amino acid sequence is displayed above the corresponding nucleotide sequence with standard one-letter amino acid code. Amino acid no. 1 is assigned to the first amino acid residue of the mature γ subunit and marked with a *. The amino acid residues in the signal peptide are indicated by negative numbers. The four hydrophobic membrane-spanning regions are marked as M1-M4. The potential sites for asparagine N-glycosylation are indicated by vertical arrows.

nucleotide chain termination method (see Materials and Methods).

The complete nucleotide sequence of clone M169 is shown in Figure 2. It has an open reading frame of 1,557 bases flanked by 11 bases on the 5' side and 148 bases on the 3' side. The 3' untranslated region is followed by a stretch of adenosine residues (not shown in Figure 2), presumably copied from the mRNA polyadenosine tail during reverse transcription. Beginning 18 bases 5' to the polyadenosine stretch, there is a consensus polyadenylation signal AATAAA (24,25). Thus the insert appears to contain the complete 3' untranslated region of the mRNA.

The open reading frame of clone M169 has a methionine codon ATG at the third codon position following an in-frame termination codon TGA (Figure 2). If this methionine codon is used as the initiation codon, the translated polypeptide chain would consist of 519 amino acid residues. When this putative polypeptide was compared with the published AChR γ subunit sequences from other species, a high degree of homology was revealed (see below). On this basis, we assign the protein coded by clone M169 to be the mouse muscle AChR γ subunit. Using the *Xenopus* oocyte assay system (16), the RNA transcribed from clone M169 in an SP6 vector by *in vitro* transcription showed functional substitution for the *Torpedo* γ subunit, thus confirming the assignment (unpublished results, K. M. Mayne, K. Yoshii, L. Yu, and N. Davidson).

Structural Analysis of the Mouse Muscle AChR γ Subunit

The deduced amino acid sequence for the mouse muscle AChR γ subunit is displayed above the corresponding nucleotide sequence in Figure 2. The first 22 amino acid residues have the characteristic features of a signal peptide common to membrane-associated and secretory proteins (26-28). These features are a stretch of highly hydrophobic amino acid residues followed by a hydrophilic residue (glutamine), and a residue with a short side chain located at the putative cleavage site (serine). Comparison with AChR γ sequences from other species (see below) supports the interpretation that the arginine assigned as amino acid no. 1 in Figure 2 is indeed the first amino acid residue of the mature γ subunit of the mouse AChR and that the oligopeptide preceding it is the signal peptide. This signal peptide is presumably involved in the translocation of the newly synthesized protein across the rough endoplasmic reticulum membrane. Based on the above assignment, the molecular weights of the precursor and the mature γ subunit were calculated to be 58,752 and 56,493, respectively.

The mouse AChR γ subunit has structural features common to all the AChR subunits from mouse and other species (2,11,29). It has four highly hydrophobic segments characteristic of transmembrane domains. They are designated M1-M4, and their amino acid positions are M1, 219-245; M2, 252-270; M3, 286-307; and M4, 455-473 (Figure 2).

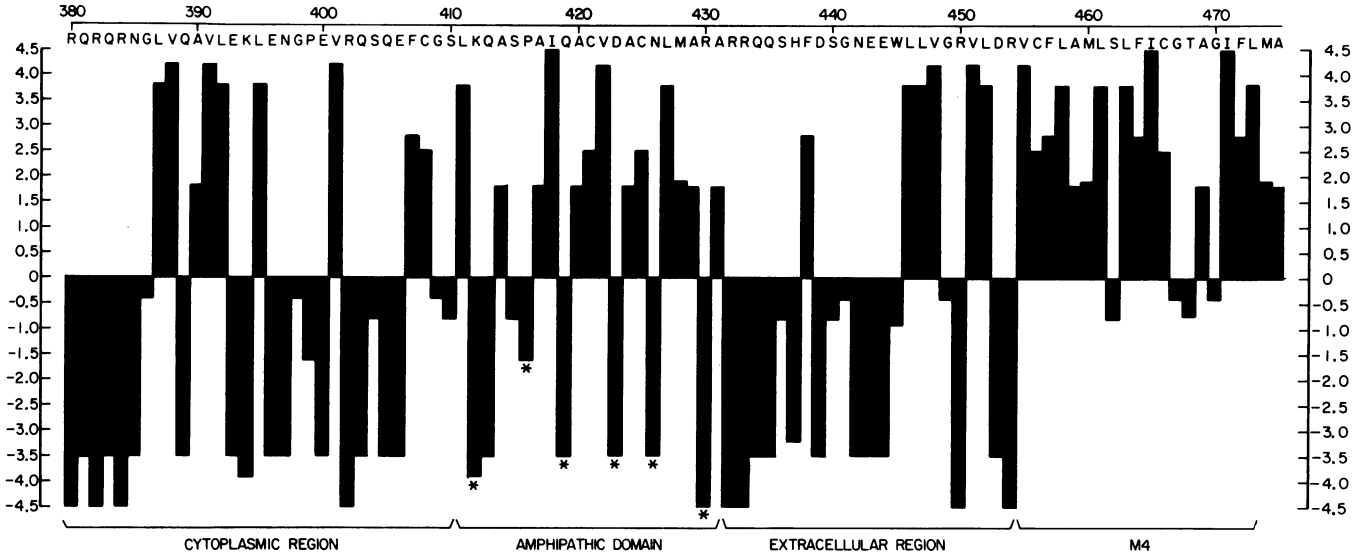

Figure 3. Analysis of the hydropathic properties of the mouse AChR γ subunit. Amino acid residues are plotted against their hydropathy index values. Positive values indicate hydrophobicity and negative values hydrophilicity. The amino acid sequence is displayed on top of the histogram and is numbered according to Figure 2. The stars mark the hydrophilic amino acid residues in the putative amphipathic membrane-spanning domain that align the inside of the ion channel.

The possible existence of a fifth membrane-spanning region, the amphipathic α-helix domain (30,31), was examined. As shown in Fig. 3, the region between amino acid residues no. 380 and no. 475 was analysed by plotting the amino acid residue numbers against their hydropathy index values (32). Positive values indicate hydrophobicity and negative ones hydrophilicity. The region between residues no. 411 and no. 431 shows a remarkable regularity of highly hydrophilic residues at the average distance of 3.5 amino acid residues while the rest of this region is largely hydrophobic, a salient feature of a membrane-spanning amphipathic α-helix. The cytoplasmic and extracellular regions on both sides of the amphipathic domain are overall hydrophilic and the membrane-spanning region M4 is highly hydrophobic (Figure 3). The hydrophilic amino acid groups in the amphipathic domain are at positions 412, 416, 419, 423, 426, and 430 with an average hydropathy index value of -3.42. The rest of this region has an average hydropathy index value of 1.81, characteristic of a hydrophobic transmembrane region. Therefore, it seems probable that this region forms an amphipathic transmembrane segment with the hydrophilic side of the α-helix contributing to the charged lining proposed for the AChR ion channel (30,31).

Many proteins, including membrane proteins, enzymes, secretory proteins without enzymatic functions, and immunoglobulins, undergo post-translational modifications to become glycoproteins by the enzymatic addition of carbohydrate chains to L-asparagine residues in the polypeptide chain (33). Analysis of many such modified proteins has revealed a consensus sequence asparagine-X-serine (threonine) where X can be any amino acid residue with the possible exception of aspartic acid (33,34). This consensus sequence is a necessary, but not a sufficient, condition for carbohydrate chain addition. There are four such potential N-glycosylation sites in the mouse AChR γ sequence (Figure 2), at positions 30, 141, 306, and 354. If the γ subunit has membrane domains as proposed, only Asn_{30} and Asn_{141} will be exposed from the membrane and thus accessible for carbohydrate attachment (35). In this interpretation, Asn_{306} is in the membrane-spanning region M3 and Asn_{354} is on the cytoplasmic side of the membrane.

Sequence Comparison of AChR γ Subunit from Different Species

cDNA and genomic clones coding for the γ subunit of the nicotinic AChR have been isolated from a number of species. To study the relatedness of the AChR γ subunit, the deduced amino acid sequence of the mouse AChR γ subunit was compared with those of human (36), calf (37), chicken (38), and *Torpedo* (4,8). As shown in Figure 4, the mouse sequence exhibits high degrees of homology with those of human and calf and somewhat lower degrees of homology with those of chicken and electric ray, indicating the close evolutionary relationship among mammals.

We have also searched for regions where, in spite of amino acid sequence

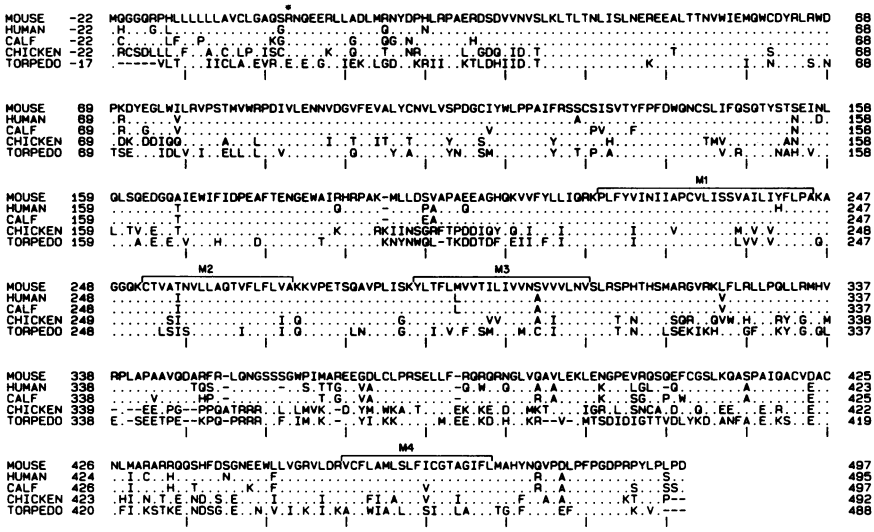

Figure 4. Comparison of the amino acid sequences for the nicotinic AChR γ subunit precursors of mouse, human, calf, chicken and electric ray *Torpedo californica*. The protein sequences are aligned with respect to the first amino acid residues in the mature proteins (marked by a *), and the amino acid residues in the signal peptides are given negative numbers. A dot indicates identity with the mouse sequence at that position. Gaps inserted to allow maximal homology are represented as dashes. The hydrophobic membrane-spanning regions are marked M1-M4.

divergence, functional features are conserved. For this purpose, amino acid substitutions by their functional equivalents were scored as homologous in the analysis. These functionally equivalent groups are: acidic, D and E; basic, H, K, and R; non-polar, A, F, I, L, M, P, Q, V, and W; and polar, C, G, N, S, T, and Y. Homology by these criteria may identify regions of conserved structure, presumably important for the assembly and function of the AChR. It is of course a mathematical necessity that the homology value between two sequences will be enhanced after the functional substitution. Nevertheless, the degree of sequence homology between the evolutionarily more distant sequences from this comparison is quite striking. As shown in Table 1, the 67% overall homology between the actual sequences of mouse and chicken AChR γ subunits is increased to 83% and the 56% between mouse and *Torpedo* to 77% after the functional group substitution. A still more striking conservation was revealed when the membrane-spanning regions were analyzed. The homology values between the actual sequences are significantly higher for the transmembrane regions than those for the entire γ subunits and, with allowance for functional substitution, these membrane segments all showed over 90% homology with each other. Both the divergence and the conservation of transmembrane regions have been reported before

TABLE 1
Amino acid sequence homology of AChR γ subunit
between mouse and human, calf, chicken, and *Torpedo*

		Human	Calf	Chicken	*Torpedo*
Homology over entire precursor	Actual sequence	90%	90%	67%	56%
	After functional substitution	93%	95%	83%	77%
Homology for M1-M4*	Actual sequence	95%	95%	80%	68%
	After functional substitution	97%	98%	97%	92%

* The sum of M1, M2, M3, and M4 as aligned in Figure 4.

(39,40). When the protein sequences were compared for class I and class II antigens of the major histocompatibility complex, it was found that the transmembrane regions are more divergent than the rest of the proteins for class I antigens (39) while they are more homologous for class II antigens (40). Since class I antigens are monomeric protein molecules and class II antigens are dimers, it may be considered that the high degree of homology for the class II antigen transmembrane domain is necessary for the interaction between the heavy and light chains to form a functional class II molecule and that there is no such evolutionary pressure for class I antigens. Our result also supprts this line of thinking and suggests that these putative membrane-spanning regions have been correctly identified and play an important role in the assembly and function of the AChR. The conservation of the membrane-spanning regions in AChR α subunits across species has been noticed before (15).

A novel γ-like subunit of AChR, the ε subunit, has been identified from a cDNA sequence from calf muscle (41). When it was compared with the mouse γ subunit, a homology of 53% was obtained. Clearly the mouse γ subunit is more homologous to the calf muscle γ subunit (90% homology).

The mouse AChR γ subunit contains two cysteine residues at positions 128 and 142 that are also conserved in the γ subunit from other species (Figure 4) as well as in all the other known subunits (11). These cysteine residues may be involved in the formation of a disulfide bridge (4).

Previously, our laboratory reported the isolation of a mouse cDNA clone by the hybridization to a *Torpedo* γ subunit cDNA probe. Sequence analysis showed that the protein coded by this clone exhibits a slightly higher degree of homology with the *Torpedo* δ subunit than with γ and thus it was tentatively assigned as a mouse AChR δ

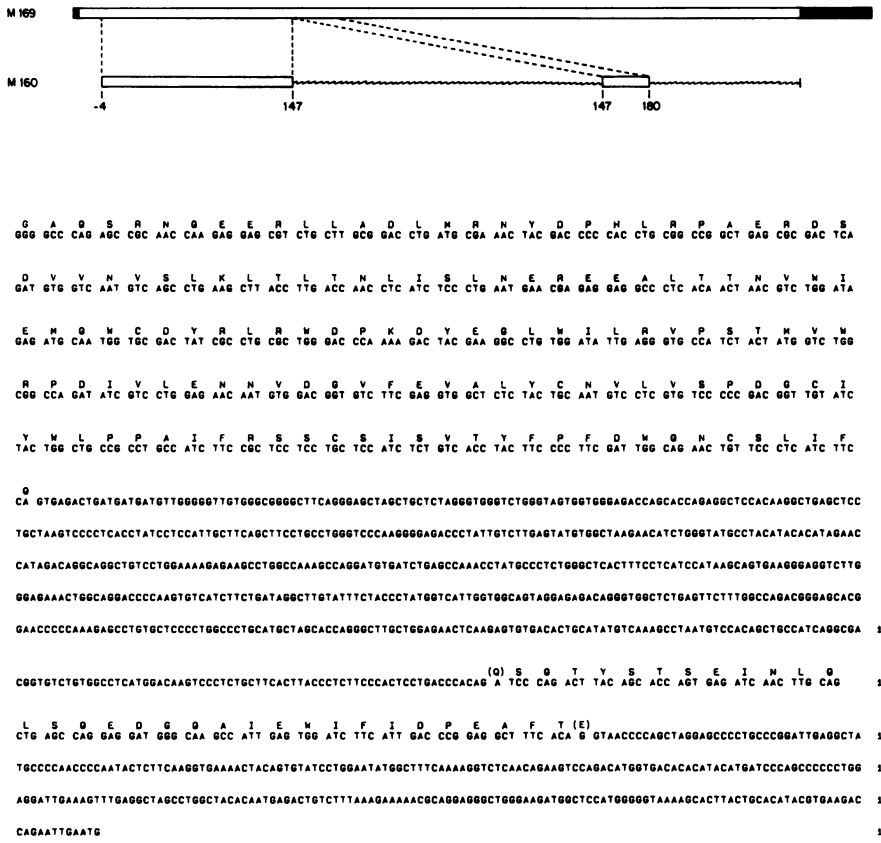

Figure 5. Mouse AChR γ subunit intervening sequences. Clone M160 is aligned with respect to the mature message clone M169 in the top panel. The open boxes represent protein coding regions, and the closed boxes in M169 indicate the 5' and 3' untranslated regions. The wavy lines in M160 represent intron sequences. The numbers at the coding region boundaries below M160 denote the amino acid positions as defined in Figure 2. DNA sequence of M160 and the translation of exons are shown in the bottom panel. Nucleotide no. 1 corresponds to nucleotide no. 55 of the M169 sequence in Figure 2. Amino acid sequences of the protein coding sections are displayed above the DNA sequence and are numbered according to Figure 2.

subunit cDNA (14). Subsequent functional analysis indicated that the RNA made from this clone could replace the RNA of the *Torpedo* δ subunit to produce a highly functional AChR hybrid protein and that it could not substitute for the *Torpedo* γ subunit RNA (16). The isolation of the mouse AChR γ subunit cDNA has further confirmed our previous identification of the mouse AChR δ subunit cDNA clone. When the mouse γ and δ subunits were compared, they showed overall homologies of 58% at the DNA sequence level and 50% at the protein sequence level (data not shown).

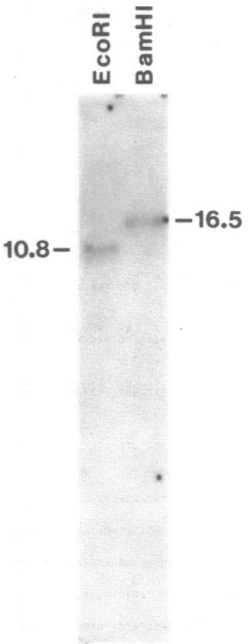

Figure 6. Genomic DNA blot analysis. 10μg of BC3H-1 DNA was digested either with *Eco*RI or *Bam*HI, size fractionated by agarose gel electrophoresis, blotted, and hybridized with nick-translated full-length M169 DNA. The sizes of the hybridizing bands are given in kilobases.

Partially Processed RNA Molecules

There are two clones that were selected from the original cDNA library by screening with the *Torpedo* γ probe that showed homology with clone M169 over some portion but not over others. One of these clones, M160, was analyzed by sequence determination. The arguments presented below lead to the conclusions that the sections in M160 nonhomologous to M169 are intron sequences and that we have isolated a clone derived from a partially processed nuclear RNA. The published genomic sequences for the AChR γ gene of human (36) and chicken (38) both have introns disrupting a codon for glutamine at position 147 and disrupting a codon for glutamic acid at position 180. When the sequences of M160 and M169 were compared, it became obvious that M160 codes for part of the AChR γ subunit with the codons for Gln_{147} and Glu_{180} interrupted by two non-coding sequences (Figure 5A). These sequences have nonsense codons in all three reading frames and are flanked by dinucleotides GT on the 5' side and AG on the 3' side (Figure 5B), the two major characteristics of intervening sequences (42,43). Therefore, we conclude that these two non-coding sequences are indeed intron sequences. The human and chicken AChR γ genes have a total of 11 introns interrupting their coding regions at identical positions. Because the two introns in M160 are present at the same positions as in the human and chicken genes, it is reasonable to believe that the same overall exon-intron structure exists for the mouse

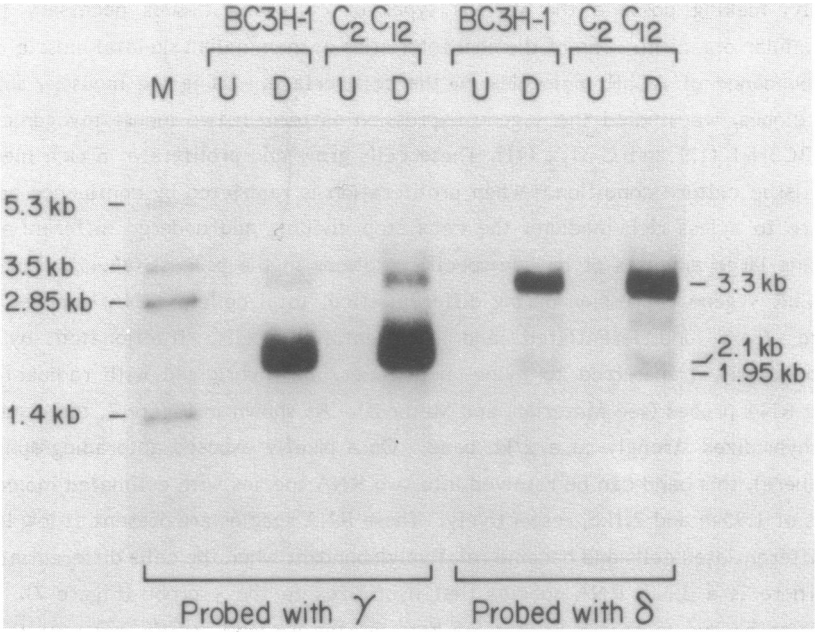

Figure 7. RNA blot hybridization. 10μg of total cellular RNA from appropriate cells were fractionated in each lane, blotted, and hybridized with ^{32}P-RNA probes. U, undifferentiated cells; D, differentiated cells. M, RNA markers made by *in vitro* SP6 transcription of mouse γ cDNA clone. Kb, kilobases.

gene. However, the protein coding region in M160 between codon -4 and codon 147 is continuous, lacking the three introns that are present in both human and chicken genes (Figure 5). Therefore, we propose that clone M160 was derived from a partially processed nuclear RNA molecule coding for the mouse AChR γ subunit. Intron-containing cDNA clones have been reported before for the calf AChR γ subunit (37).

The Gene Coding for the AChR γ Subunit

The AChR γ subunit is encoded by a unique gene in the chicken genome (38) and perhaps also a single gene in the human genome (36). For calf and *Torpedo* γ subunit, there is no available information regarding the gene number. To estimate the number of gene(s) coding for the mouse AChR γ subunit, genomic DNA blot analysis was performed using the full-length M169 DNA as the hybridization probe. As shown in Figure 6, *Eco*RI and *Bam*HI digested DNA each gives a single hybridizing fragment. This simple pattern of hybridization suggests that the mouse SChR γ subunit is probably encoded by a single gene.

γ Gene Expression in Murine Myogenic Cell Lines

The activity of the majority of eukaryotic genes is regulated both temporally and

spatially, making possible the various types of cells and tissues necessary for a multicellular organism. One of the characteristics of mammalian skeletal muscle is the high abundance of AChR molecules on the cell surface. Using the mouse γ subunit cDNA clones, we studied the γ gene expression pattern in two mouse myogenic cell lines, BC3H-1 (12) and C_2C_{12} (41). These cells grow and proliferate in rich medium under tissue culture condition. When proliferation is restricted by confluence and/or exposure to a less rich medium, the cells stop dividing and undergo differentiation, producing large amounts of muscle-specific proteins in the process (44,45). To study the AChR γ gene expression during differentiation, total cellular RNA samples were isolated from undifferentiated and differentiated cells, fractionated by gel electrophoresis, transferred to nylon membranes, and hybridized with radioactively labeled RNA probes (see Materials and Methods). As shown in Figure 7, the γ subunit probe hybridizes strongly to a 2 kb band. On a briefly exposed autoradiograph (not shown here), this band can be resolved into two RNA species with estimated molecular lengths of 1.95kb and 2.1kb, respectively. These RNA species are present at low levels in undifferentiated cells and become relatively abundant when the cells differentiate.

There is a 3.3kb RNA species that hybridizes to the γ probe (Figure 7). This appears to be due to crosshybridization between the γ probe and the message for the AChR δ subunit, as indicated by the strong hybridization at the same position to the mouse δ probe (14) in Figure 7. The δ probe also cross-hybridizes weakly with the two γ messages.

The absolute abundance of the γ messages in total cellular RNA was estimated by comparison of the signal intensity on RNA blots between the band of a 1.4kb RNA standard and the γ mRNA bands (Figure 7). The standard was generated by ***BglI*** restriction digestion of the γ cDNA sequence M169 subcloned into the expression vector pIBI76 and subsequent *in vitro* transcription with SP6 RNA polymerase (see Materials and Methods). The radioactive precursor [^{32}P-α]CTP was used as a tracer (1:3.5x10^4 dilution with non-radioactive CTP). The purity of the transcription product was ascertained by running an aliquot of the RNA in a gel and exposing the gel to an X-ray film. The *in vitro* transcription efficiency was calculated and the RNA concentration derived. 10pg of this *in vitro*-generated γ subunit RNA were mixed with other RNA size markers, subjected to gel electrophoresis side-by-side with 10μg of total cellular RNA samples from undifferentiated and differentiated cells, blotted to nylon membranes, and hybridized to the antisense γ probe (Figure 7). Thus, the 1.4kb RNA serves not only as an RNA size marker, but also as a mass standard for the AChR γ messages. The intensities of the RNA bands were measured by densitometry tracing, the area under each peak integrated by a graphic digitizer, and the values normalized to the standard. The results are shown in Table 2. The mRNA abundance for the AChR γ

TABLE 2
Quantitation of the mouse AChR γ mRNA

Source of RNA	SP6 RNA	BC3H-1 cells		C_2C_{12} cells	
		Undifferentiated	Differentiated	Undifferentiated	Differentiated
Quantity of RNA	10pg	10μg	10μg	10μg	10μg
Signal intensity (normalized to SP6 RNA)	100%	49%	1380%	72%	3570%
Message ratio		1 : 28		1 : 50	
Message abundance (pg/μg cellular RNA)		0.49	13.8	0.72	35.7

subunit is very low before differentiation and is dramatically increased when the cells are differentiated, reaching 13.8pg per μg total cellular RNA in BC3H-1 and 35.7pg in C_2C_{12} cells. The induction ratio is approximately 28-fold for BC3H-1 and 50-fold for C_2C_{12}. This up-regulation of AChR γ messages upon differentiation correlates well with the observation that the large amounts of AChR molecules appearing on the muscle surface during differentiation were synthesized *de novo* rather than being stored in the cytoplasm before differentiation (45-47) and suggests that the control of expression is mainly at the transcriptional level.

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While this manuscript was in the final stages of preparation, we received a copy of a preprint from Dr. J. Boulter and coworkers in the laboratory of Drs. S. Heinemann and J. Patrick at the Salk Institute describing their independent isolation and sequence determination of a γ subunit cDNA clone derived from BC3H-1 cells. There are only a few minor disagreements between the two sequences. We are grateful to these authors for sharing their results prior to publication.

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